By-passing selection: direct screening for antibody–antigen interactions using protein arrays

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ABSTRACT

We have developed a system to identify highly specific antibody–antigen interactions by protein array screening. This removes the need for selection using animal immunisation or *in vitro* techniques such as phage or ribosome display. We screened an array of 27 648 human foetal brain proteins with 12 well-expressed antibody fragments that had not previously been exposed to any antigen. Four highly specific antibody–antigen pairs were identified, including three antibodies that bind proteins of unknown function. The target proteins were expressed at a very low copy number on the array, emphasising the unbiased nature of the screen. The specificity and sensitivity of binding demonstrates that this 'naive' screening approach could be applied to the high throughput isolation of specific antibodies against many different targets in the human proteome.

INTRODUCTION

Traditionally, antigen-specific antibodies have been isolated by subjecting a 'naive' repertoire of antibodies to alternate rounds of selection by binding the target antigen and proliferation of binding clones. Selection may be *in vivo*, by injecting antigen into an animal and eliciting an immune response (1–3), or *in vitro* (4), using phage (5,6) or ribosome display (7). There are several disadvantages of such selective techniques. First, the application of selective pressure often results in the exertion of biases which mean that only a small proportion of binding antibodies are isolated from the starting repertoire. These biases could be due to selection for the binding strength of the antibody–antigen interaction itself or for other factors such as the expression level of the antibody, its toxicity to the host organism or its folding and stability characteristics. In addition, where the starting library contains many specific antibodies to many different epitopes on the antigen, selection often yields a single specific antibody to a single epitope, a phenomenon known as epitope dominance. In selections against complex antigens, consisting of many different target proteins, the problem is compounded as successive rounds of selection lead to enrichment for antibodies that bind the most frequently occurring epitopes in the mixture (8). These epitopes are usually on abundant proteins, which are likely to have been previously characterised and have known function.

Here, we have circumvented these problems by avoiding the selection step altogether. By screening an array of 27 648 human proteins with unselected antibodies, we identified a number of highly specific antibody–antigen interactions, thus by-passing animal immunisation, phage or ribosome display. Such 'naive' screening is well suited to the identification of antibodies against a wide range of target antigens with unknown or ill-defined functions. Furthermore, since this approach removes the need for the binding and proliferation steps involved in selection, it would be suitable for the automated isolation of antibodies on a proteomic scale.

MATERIALS AND METHODS

Preparation of scFvs

Recombinant single chain Fvs (scFvs) were taken from two different antibody libraries. Both libraries are based on a single human framework for V_H (V3-23/DP-47 and J_H4b) and V_κ (O12/O2/DPK9 and $J_\kappa1$), with side chain diversity (either NNK or DVT encoded libraries J and I, respectively) incorporated at positions in the antigen binding site that make contacts to antigen in known co-crystal structures and are highly diverse in the mature repertoire (18 different amino acid positions in total) (I.M.Tomlinson and G.Winter, manuscript in preparation). This fold is frequently expressed *in vivo* (9) and binds the generic ligands Protein L and Protein A, which facilitate the capture and/or detection of the antibody fragments without interfering with antigen binding. Protein A (10) is a bacterial superantigen that binds to the V_H domain (11,12) and Protein L is a bacterial superantigen that binds to the V_κ domain (13). Recombinant Protein L (Affitech, Oslo, Norway) is a tetramer and thus as a secondary reagent coupled to HRP allows much more sensitive detection than that afforded by conventional anti-tag antibodies (14–17). The expression vector we used (pIT2) is derived from pHEN1 (18) and contains a lac promotor and a pelB leader sequence upstream of the V_H-$(G_4S)_3$-V_L insert, which is then followed by His_6 and myc tags, an amber stop codon

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and the gene encoding the pIII phage coat protein. Thus, in a suitable non-supressor strain (HB2151), addition of isopropyl-thio-β-D-galactoside (IPTG) induces only scFv and not scFv–pIII fusion expression. ScFv is then directed to the periplasm and diffuses out into the supernatant. Bacteria were grown with shaking overnight at 37°C in 2× TY medium containing 100 µg/ml ampicillin and 1% glucose, then diluted 1:100 in fresh 2× TY containing 100 µg/ml ampicillin and 0.1% glucose for 3 h with shaking at 37°C. IPTG was added to a final concentration of 1 mM and the cultures were incubated with shaking overnight at 30°C. Bacteria were pelleted by centrifugation and the supernatant containing the scFvs was filtered through a 0.45 µm filter. ScFvs were tested for expression level using a Protein L, Protein A–HRP sandwich ELISA. Briefly, an immunoplate (Maxisorp; Nalge Nunc International, Rochester, NY) was coated overnight with 100 µl/well 1 µg/ml Protein L in phosphate-buffered saline (PBS), blocked for 3 h in 2% Marvel PBS (MPBS), washed three times in PBS and incubated for 1 h with 100 µl/well scFv supernatant (1 in 4 dilution in 2% MPBS). The plate was washed three times in 0.05% Tween-20 PBS (PBST) and incubated for 1 h with 100 µl/well 1:2000 Protein A–HRP conjugate (AP Biotech, Uppsala, Sweden) in MPBS. The plate was washed three times in PBST and developed with 100 µl/well substrate solution [100 µg/ml TMB (3,3′5,5′-tetramethylbenzidine in dimethyl sulphoxide) in 100 nM sodium acetate, pH 6, with 10 µl of 3% hydrogen peroxide added directly before use]. After 5 min incubation, the reaction was stopped with 50 µl/well 1 M sulphuric acid and the plate read at OD 450–650.

Filter preparation and hybridisation conditions

The protein array consists of a PVDF membrane spotted with 27 648 clones from human foetal brain cDNA expression library hEx1 (19,20). The recombinant proteins in this array are expressed in the bacterial cytoplasm and then released by lysis of the cells (which simultaneously denatures the protein) using NaOH. This array can therefore only be used with molecules that are able to recognise denatured protein, such as antibodies. For probing with scFv, hEx1 membranes obtained from the Resource Center of the German Human Genome Project (http://www.rzpd.de) were soaked in absolute ethanol for 20 min and then washed for 5 min in 1 l of 0.1% Tween-20, 1% Triton PBS (PBS-T-T). Each membrane was rinsed twice in 1 l of PBS and washed for 5 min in a further 1 l of PBS. For blocking, membranes were then incubated for 45 min in 1 l of 3% MPBS, followed by 1 h incubation in 60 ml scFv supernatant mixed with 60 ml 2× PBS, 6% Marvel. Membranes were then washed for 5 min in 1 l of PBS-T-T, followed by two rinses each in 1 l of PBS and one 5 min wash in 1 l of PBS. Each membrane was then incubated for 40 min in 150 ml of a 1:2000 dilution of Protein L–HRP in 3% MPBS, washed twice for 5 min in 1 l of PBS-T-T and then twice for 5 min in 1 l of PBS. The filters were then developed in a total volume of 60 ml of ECL reagents (AP Biotech, Uppsala, Sweden) and exposed to photographic film (Kodak, Rochester, NY). All incubations and washes were performed with gentle agitation.

RESULTS

High density protein arrays, constructed using cDNA from human foetal brain, have previously been probed with mouse monoclonal antibodies and shown to identify their cognate proteins, GAPDH and HSP90α (19). In order to check whether the same approach would work with recombinant antibody fragments, an anti-ubiquitin scFv (UBI B1), which had previously been selected by phage display against purified ubiquitin (I.M.Tomlinson and G.Winter, manuscript in preparation), was used to probe the same arrays (data not shown). Seven cognate proteins were identified and several of these were sequenced. All were found to contain poly-ubiquitin repeats, verifying that scFvs specifically bind their target antigen and can be detected using this system.

For naive screening, 48 unselected clones from each of the NNK and DVT libraries were picked into a microtitre plate, grown, induced and screened for scFv expression. The six highest expressing clones from each library were chosen and a large volume of supernatant containing scFv was produced. Highly expressing scFvs were chosen as these would be most likely to give a strong signal on the array if a cognate antigen was present. These supernatants were mixed to create two separate pools, each containing six different scFvs and two duplicate protein arrays were probed. A small number of cDNA clones (14 in total) were found to be positive on one of the two filters (Fig. 1) but not both, indicating specific binding. These clones were picked and lysates from each were separately run out on polyacrylamide gels and then transferred by western blotting to PVDF membranes. Each lysate was separately probed with each of the 12 scFvs. In total, four different specific antibody–antigen interactions were confirmed (Table 1). The antibody sequences confirmed that two of the scFv clones came from the NNK library (G12 and H11) and two were from the DVT library (D12 and C2). Sequencing of the cDNAs indicated that three are of unknown function, whereas the fourth is translated in a different frame

Table 1. Antibody–antigen interactions

Antigen	RZPD name	Accession no.	Antigen size	ScFv	CDR H2	CDR H3	CDR L2	CDR L3
M	B12492Q3	AJ297364	21.1 kDa	D12	**G**I**TYSGDATS**YADSVKG	**AYTS**FDY	**N**ASN**L**QS	QQ**YNATPGT**
B	F10260Q003	AJ297362	12.0 kDa	G12	**R**I**DPTGNITS**YADSVKG	**VAEI**FDY	**R**AS**R**LQS	QQ**ALSLPTT**
C	C04260Q003	AJ297363	14.0 kDa	H11	**SITLAGASTS**YADSVKG	**ASRS**FDY	**N**ASSLQS	QR**ISPRPTT**
O	M05499Q3	AJ297365	8.8 kDa	C2	**GIYASGSTTA**YADSVKG	**AATS**FDY	**S**AS**Y**LQS	QQ**ATTSPNT**

The antigen sizes were calculated from DNA sequence data (including the N-terminal MRGSHHHHHHGSYLGDTIESSTHAS tag). Residues in bold indicate those positions in the scFv that were diversified in the NNK and DVT libraries. RZPD clone names omit the prefix MPMGp800.

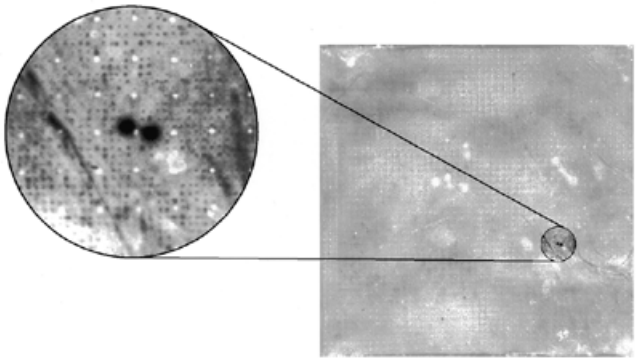

Figure 1. Detection of specific antibody–antigen interactions on the hEx1 cDNA array. Bound scFv is detected with Protein L–HRP. Each cDNA was double spotted to aid identification of positives. The enlarged area shows the detection of unknown antigen M with scFv D12.

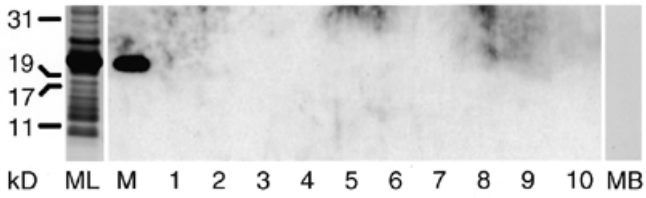

Figure 2. Western blotting analysis indicating the specificity of scFv D12 for its target, antigen M. ML is the bacterial lysate containing recombinant antigen M, stained with Coomassie blue. Lane M and lanes 1–10 are from a western blot probed with scFv D12. Antigen M gives a strong signal at ~21 kDa, whereas all the the other antigens (1–10) are negative. Antigens 1–10 are hen egg lysozyme, bovine serum albumin, α-chymotrypsinogen, thyroglobulin, turkey egg lysozyme, avidin, glycophorin, ovine submaxillary mucin, peanut biotin and amarantus biotin, respectively. Lane MB is from a western blot of antigen M probed with a irrelevant scFv BiP A4 which does not bind antigen M.

from a known protein. This protein is deposited twice in GenBank, as gClq-R (21) (accession no. X75913) and as SF2p32 (22) (accession no. L04636).

One of the pairings between an antibody and an unknown antigen (scFv D12 and antigen M) was further characterised. The specificity of scFv D12 for antigen M was confirmed by probing similar concentrations of M and 10 other antigens with the anti-M scFv D12 (Fig. 2). Only antigen M was detected. A control scFv from the NNK library which was selected by phage display against immunoglobulin binding protein (BiP) did not detect antigen M (Fig. 2). In order to demonstrate the utility of scFvs isolated by naive array screening, the sensitivity of detection of scFv D12 for antigen M was compared to the sensitivity of detection of the scFv UBI B1 (a phage-selected antibody) for ubiquitin. Equal amounts of the two recombinant lysates (which express equivalent levels of recombinant protein) were run on a gel and western blotted with their respective scFvs. A similar detection threshold of 50 ng protein was seen for both M and ubiquitin using scFv

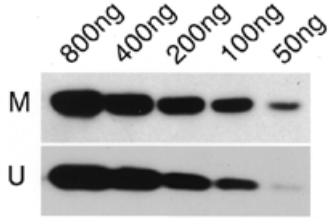

Figure 3. Western blotting analysis indicating the sensitivity of detection of scFv D12 for its target, antigen M. The sensitivity of detection of D12 for antigen M is compared to the sensitivity of detection of the phage-selected scFv UBI B1 for ubiquitin. In both cases, secondary detection is with Protein L–HRP.

supernatants (Fig. 3). This threshold was reduced for both antibody–antigen pairs to sub-nanogram levels when purified scFvs were used instead of supernatants (data not shown). The array and phage screened scFvs therefore detect their respective target antigens with a similar sensitivity.

DISCUSSION

Although several large 'naive' antibody libraries are available (23–27), libraries containing as few as 10^7 or 10^8 members can also be used to isolate antibodies against almost any protein antigen using phage selection (25,28–31). Similarly, the primary mouse antibody repertoire is ~5×10^8 in size and will elicit an immune response to most non-self proteins. In both cases, selection yields a handful of specific antibodies which are generally in the micromolar range and these can be improved by subsequent affinity maturation [either *in vivo* (32,33) or *in vitro* (34–37)]. Thus, relatively small repertoires contain antibodies to any given target, even though the potential diversity of targets is huge. This indicates that a given antibody, no matter how high the affinity for its selected target, must bind structurally unrelated targets (38). As a consequence, a 'specific' antibody is only truly specific in the background in which it is tested. Thus, if a therapeutic antibody must target a cell surface protein, provided it does not bind any other protein in the serum, it is sufficiently 'specific' for the task.

We wondered whether we could exploit the ability of antibodies to bind specifically to multiple targets as a means of isolating recombinant antibodies against components of large protein arrays. Instead of using antibodies previously selected against antigen, we chose to use semi-synthetic antibodies that have never been selected against any target antigen and are therefore truly 'naive'. Highly expressed (but unselected) antibodies from a new semi-synthetic library (I.M.Tomlinson and G.Winter, manuscript in preparation) were therefore screened against a recombinant foetal brain protein array (19,20) and from only 12 antibodies tested, cognate proteins in the array were identified for four of them. None of these antibodies bound any other proteins in the array (as demonstrated in the initial screen) and none bound to any endogenous bacterial proteins at a detectable level. In addition, all of the four antibody–antigen pairs were unique in that each of the antibodies bound to a different antigen and all of the four antigens bound

a single antibody. This level of specificity and the strength of detection indicates that the affinities are probably in the micromolar range.

One practical advantage of our screening approach, over selection-based approaches, is that any positive interactions that are within the detection limits of the system have the potential to be identified. Furthermore, if a particular protein is present on the cDNA array, then the number of times it occurs there (and therefore its abundance within the source tissue) does not affect the probability of finding an antibody which binds to that protein. This is useful for identifying antibodies that bind proteins of lower abundance. Another advantage is that screens with different antibodies (or mixtures of antibodies) can easily be performed in parallel, a feature facilitated by the use of robotics. To date, the high throughput identification of specific antibodies has been hampered by the need for *in vivo* or *in vitro* selection. The immunisation of thousands or tens of thousands of mice followed by the production of hybridomas from each is not practical (nor is it ethically acceptable). Phage and ribosome display require washing steps and liquid proliferation for subsequent rounds of selection, which would rely heavily on expensive high throughput liquid handling. Our method, however, makes use of much simpler bacterial transfer robotics to create the arrays, which can then be probed simply by immersion in baths containing the various reagents. Even if selection were to be used to enrich for binding antibodies, there would still need to be a mass screen to isolate individual binding clones and these would then need to be checked for cross-reactivity. The naive screening approach we have developed combines the initial identification of binding pairs with a screen to confirm specificity in a single step.

In order to calculate the precise 'hit rate' of our naive screen, we need to determine the actual number of different protein targets in the array. Although the array contains 27 648 members, since it is not normalised, many of them are likely to represent the same, or highly related, gene transcripts. Indeed, we found that 7 of 27 648 corresponded to ubiquitin and Büssow *et al.* (19) showed that 0.07% were HSP90 (56/80 640) and 0.26% (206/80 640) were GAPDH. Furthermore, only 19.6% were found to be reasonably well expressed (20). It is therefore our estimate that the array actually contains between 1000 and 5000 different expressed polypeptides. Thus, since one in three antibodies tested identified a cognate protein, a single detectable interaction was observed for every 3×10^3–1.5×10^4 different antibody–antigen combinations screened. When compared to phage selection of a 10^8 antibody library, which typically yields a maximum of 100 different clones (1 binder in 10^6 non-binders), the 'hit rate' achieved by direct screening is remarkably high and suggests that the isolation of antibodies from large libraries by conventional techniques may be very inefficient. Thus, many binding clones, which are specific and may be useful leads for affinity maturation, may be lost due to inherent biases in the selection processes. Furthermore, it indicates that any small repertoire containing structurally diverse surfaces may be sufficient to isolate binding entities. Indeed, one wonders how many protein pairings highlighted by yeast two-hybrid or similar screens correspond to naturally occurring interactions and how many are due to fortuitous binding of protein pairs that never encounter one another *in vivo*. Thus, high throughput screens of putative protein–protein interactions, for example yeast two-hybrid (reviewed in 39) and large scale array screening (40), may need to take account of the different cellular compartments where the protein pairs exist *in vivo*.

Our work shows that recombinant antibodies can be used to rapidly identify their cognate antigens on high density arrays of denatured protein. Arrays of this type will therefore be particularly useful where the binding specificities of phage- or ribosome-selected antibodies are not known, for example, where the selection was performed using a cellular extract. More importantly, we have demonstrated that truly naive repertoires can be used to identify specific antibody–antigen interactions by screening high density arrays of target proteins. Of course, one could envisage reversing the screen, thus creating a large antibody array and probing it with a given protein. Whilst this would itself generate a number of technical hurdles, for example, how to stably immobilise native antibodies on a solid support, our results suggest that an array in the range of 10^4–10^5 different functional antibodies should be sufficient to isolate specific antibodies of moderate affinity to any given target antigen (which could, if required, be affinity matured). Alternatively, one could imagine using unselected antibodies to probe naturally compartmentalised antigens, for example in tissue samples or whole organisms such as *Drosophila melanogaster*. Regardless of the precise format, we believe that naive screening will enable parallel large scale identification of antibodies that bind specifically to a wide range of proteins in humans or other (model) organisms. Large panels of such antibodies are likely to have applications in both research and medical diagnostics where an expression profile of a wide variety of proteins would be useful.

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